

## Anti-inflammatory strategies for homocysteine-related cardiovascular disease

Chih-Pei Lin<sup>1,2,7</sup>, Yung-Hsiang Chen<sup>6</sup>, Hsin-Bang Leu<sup>3,5</sup>, Shing-Jong Lin<sup>3,5,8</sup>, Yuh-Lien Chen<sup>9</sup>, Song-Lih Huang<sup>2</sup>, Jaw-Wen Chen<sup>4,5,8</sup>

<sup>1</sup> Faculty of Biotechnology and Laboratory Science in Medicine, Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei, Taiwan, <sup>2</sup> Institute of Public Health, National Yang-Ming University, Taipei, Taiwan, <sup>3</sup> Institute of Clinical Medicine, <sup>4</sup> Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan, <sup>5</sup> Cardiovascular Research Center, National Yang-Ming University, Taipei, Taiwan, <sup>6</sup> Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan, <sup>7</sup> Department of Pathology and Laboratory Medicine, <sup>8</sup> Division of Cardiology, Taipei Veterans General Hospital, Taipei, Taiwan, <sup>9</sup> Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan

### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Homocysteine
  - 3.1. Hyperhomocysteinemia
  - 3.2. Hyperhomocysteinemia induces endothelial dysfunction
  - 3.3. Hyperhomocysteinemia and vitamin supplement
4. Antioxidative and anti-inflammatory strategies
  - 4.1. Antioxidant supplement
  - 4.2. Anti-inflammatory strategy
5. Summary and prospective
6. Acknowledgement
7. References

## 1. ABSTRACT

Homocysteine may induce vascular damage for atherosclerosis. Vitamin/folate supplementation has been proposed to reduce the cardiovascular disease risk. Nevertheless, there has no randomized clinical trial clearly proven the efficacy of reducing the homocysteine as a means of lowering the incidence of cardiovascular disease. Homocysteine induces oxidative stress leading to endothelial dysfunction. In addition, homocysteine-induced oxidative stress favors lipid peroxidation and induces production of inflammatory factors, thus accelerating atherosclerosis. In this paper, we reviewed the available evidence concerning the association between homocysteine and cardiovascular disease, with the objective of discussing the pertinence of screening, treatment, and prevention of hyperhomocysteinemia-related cardiovascular disease. Our previous findings also indicate the significant role of mononuclear cells activation in homocysteine-induced endothelial dysfunction; treatment with statins attenuated homocysteine-induced endothelial adhesiveness, indicating the novel endothelial protection effects of statins in the presence of homocysteine. Since inflammation and oxidative stress is critical to homocysteine-induced vascular damage, the inhibition of endothelial dysfunction and mononuclear cell activation by anti-inflammatory and/or antioxidative drugs/agents may serve as a potential therapeutic strategy for hyperhomocysteinemia-related cardiovascular disease.

## 2. INTRODUCTION

Cardiovascular disease, the leading cause of death worldwide, is causally related to “classical” risk factors such as elevated blood pressure, cholesterol, glucose level, and smoking (1, 2). Recently, a causal role in the development of cardiovascular disease is also suggested for numerous other independent risk factors, including an elevated plasma homocysteine (3-5), although the certain mechanisms linking homocysteine to cardiovascular disease are unclear. There still has been an ongoing debate about the relationships of elevated plasma homocysteine levels and some other proatherogenic factors those are actually responsible for the cardiovascular diseases. Vitamin/folate supplementation has been proposed to reduce the cardiovascular disease risk. Nevertheless, there has no randomized clinical trial clearly proven the efficacy of reducing the homocysteine as a means of lowering the incidence of cardiovascular disease (6).

Homocysteine may cause endothelial cell dysfunction (7), smooth muscle cell proliferation (8), and mononuclear cell activation (9) *in vitro*. However, the dosage of homocysteine given in many *in vitro* studies (up to 200 microM and more) far exceed pathological homocysteine levels in humans (6). Homocysteine may induce oxidative stress leading to impaired synthesis of nitric oxide (10, 11) and resulting in endothelial dysfunction (12, 13). In addition, homocysteine-induced

oxidative stress favors lipid peroxidation (14) and induces production of inflammatory factors such as monocyte chemoattractant protein-1, interleukin-8 (15, 16), and tumor necrosis factor- $\alpha$  (17, 18), thus accelerating atherosclerosis.

Epidemiological studies showed a graded association between plasma homocysteine levels and risks of cardiovascular disease and stroke (3, 19, 20). In our previous study, among the serial inflammatory markers evaluated, only soluble vascular adhesion molecule-1 and homocysteine tended to predict future cardiovascular events in cardiac syndrome X patients (21). Mild-to-moderate homocysteine elevations in the 12-25  $\mu\text{mol/L}$  range can be corrected with folate, vitamin B6, and B12 supplementation, however it was disappointing that several interventional studies failed to show a concomitant reduction of cardiovascular events. Thus, folic acid, vitamin B6, and vitamin B12 supplements may not be used ideally for prevention of atherothrombotic events in patients with mild to moderate hyperhomocysteinemia (22-24). In recent years a number of studies were undertaken to understand homocysteine metabolism and mechanisms of its toxicity (25). Studies that provide insight into the metabolic pathways of homocysteine, regulation strategies and negative effects of elevated level of homocysteine, are crucial for the development of a new diagnostic and therapeutic methods.

The observation studies have found an association between severe hyperhomocysteinemia and atherothrombotic vascular disease. From the *in vivo* and *in vitro* models, homocysteine may promote thrombosis through enhanced platelet activation, increased thrombin generation, impaired thrombolysis, endothelial dysfunction, increased production of hydrogen peroxide, and increased oxidation of low density lipoprotein (26). The recent published meta-analysis including 30 prospective or retrospective studies which showing that a 25% lower than usual homocysteine level is associated with an 11% lower ischemic heart disease risk (odd ratio, 0.89; 95% CI, 0.83-0.96) and 19% lower stroke risk (odd ratio, 0.81; 95% CI, 0.69-0.95) (3). Furthermore, Wald *et al.* also demonstrated the recurrent cardiovascular event will increase 16% with each increase of 5  $\mu\text{mol/L}$  in the serum homocystein concentration (27), further confirmed the serum level of homocysteine is an independent factor related to cardiovascular risk.

In this paper, we reviewed the available evidence concerning the association between plasma homocysteine and cardiovascular disease, with the objective of discussing the pertinence of screening, treatment, and prevention of hyperhomocysteinemia-related cardiovascular disease. Since inflammation and oxidative stress is critical to homocysteine-induced vascular damage, the inhibition of endothelial dysfunction and mononuclear cell activation by anti-inflammatory and/or antioxidative drugs/agents may serve as a potential therapeutic strategy for clinical hyperhomocysteinemia-related cardiovascular disease.

## 3. HOMOCYSTEINE

### 3.1. Hyperhomocysteinemia

Homocysteine, a sulphur-containing amino acid, is an intermediate product in the normal biosynthesis of the amino acids methionine and cysteine. Homocysteine is predominantly metabolized from two pathways (28). The enzyme  $\text{N}^5$ ,  $\text{N}^{10}$ -methylenetetrahydrofolate reductase converts homocysteine to methionine. If  $\text{N}^5$ ,  $\text{N}^{10}$ -methylenetetrahydrofolate reductase activity is insufficient, homocysteine transformation to methionine will be impaired and homocysteine will be accumulated. The activity of  $\text{N}^5$ ,  $\text{N}^{10}$ -methylenetetrahydrofolate reductase is strongly dependent on the presence of the two vitamins, folic acid (vitamin B9) and cobalamin (vitamin B12) (29). The conversion of homocysteine to cysteine is catalyzed by cystathionine beta-synthase, an enzyme with pyridoxine (vitamin B6) as an essential cofactor. Similarly to  $\text{N}^5$ ,  $\text{N}^{10}$ -methylenetetrahydrofolate reductase, insufficient cystathionine beta-synthase activity translates into increased concentrations of homocysteine (30).

Homocysteine has been recognized as an independent risk factor for atherosclerotic diseases since elevated plasma levels of homocysteine may predict future cardiovascular mortality in clinical settings (31, 32). Although reduction of plasma homocysteine might reduce cardiovascular events after coronary intervention (33), most of the recent trials failed to demonstrate favorable outcomes by lowering homocysteine concentration with B vitamins and folic acid, or both, in patients with various atherosclerotic diseases (22, 23, 34, 35). Homocysteine was shown to enhance the endothelial adhesiveness to monocytes from healthy subjects, an *in vitro* sign of atherogenesis (9), further clinically related studies are required to clarify the pathological role of homocysteine in human atherosclerotic diseases. Besides, the alternative strategies other than homocysteine-lowering should focus on direct vascular protection in the presence of hyperhomocysteinemia (36-38).

Two types of hyperhomocysteinemia in clinical are (1) rare, severe forms due to major genetic mutations of the enzymes implicated in homocysteine metabolism; and (2) the more common forms result in moderately elevated homocysteine related to a pathogenesis that includes genetic and environmental factors. The homozygous mutation of  $\text{N}^5$ ,  $\text{N}^{10}$ -methylenetetrahydrofolate reductase and cystathionine beta-synthase can cause severe hyperhomocysteinemia. When untreated, a vascular event and premature cardiovascular disease occur in about half of these patients before the age of 30 (39, 40). Low intakes of folate, vitamin B12, and to a lesser extent vitamin B6 are associated with increased homocysteine levels independently of any genetic mutation. In addition, several diseases such as diabetes, psoriasis, cancer, and renal and thyroid dysfunction, may be associated with moderately elevated homocysteine concentrations. Finally, various drugs (folate or vitamin B6 antagonists), alcohol, tobacco, and coffee consumption, as well as older age and menopause, are also associated with high plasma homocysteine levels (6, 41).

The prevalence of hyperhomocysteinemia and mean homocysteine levels vary significantly between populations, and probably depend on diet, age, and genetic background. Most studies indicate prevalence of hyperhomocysteinemia above 10% in developed countries (6). Data from case-control and cohort studies has been gathered which supports a relationship between moderately elevated homocysteine levels and the risk of vascular disorders (coronary heart, cerebrovascular, and peripheral artery diseases) (3, 42). Recently, hyperhomocysteinemia also has been shown to be associated with a higher risk of venous thrombosis (43). In addition, dementia, depression, retinal artery thrombosis, acquired hypercoagulable states after renal transplant, thrombosis in hemodialysis patients, Parkinson disease, thrombosis in diabetic patients, and acquired thrombophilia in systemic lupus erythematosus are among published disorders associated with hyperhomocysteinemia (44).

### 3.2. Hyperhomocysteinemia induces endothelial dysfunction and mononuclear cell activation

*In vitro* studies indicate that homocysteine may have a harmful effect on endothelial cells (45). Homocysteine with pro-oxidative property may lead to impaired synthesis of nitric oxide and other vasoactive substances, resulting in endothelial dysfunction (11-13). Increasing evidences suggest that endothelial dysfunction plays a major role in the mechanisms by which homocysteine damages the blood vessel wall and induces atherosclerosis (46). Homocysteine contributes to the pathogenesis of atherosclerosis through altering endothelial functions, including injury to endothelial cells (47), activating coagulation factor V (48), modulating of tissue plasminogen activator binding to its endothelial cell membrane receptor (12), decreasing the production of nitric oxide (11, 49), and increasing oxidative stress through upregulating a nox 1-based NAD (P)H oxidase (50). Moreover, homocysteine increases cell adhesion molecule mediated leukocyte adhesion to endothelial cells (9, 36, 37, 49, 51, 52). In mice with a genetic deficiency of cystathionine beta-synthase, hyperhomocysteinaemia was associated with abnormal lipid metabolism (53) and impaired endothelial function (54). Furthermore, increased homocysteine levels could also cause an imbalance in coagulation/thrombolytic factors towards a prothrombotic state (43).

A number of studies showed that hyperhomocysteinemia impairs endothelial-dependent vasodilation, which is regarded as an early and preclinical sign of atherosclerosis (55-57). Homocysteine causes oxidant stress by effects on cellular respiration, leading to oxidation of low-density lipoprotein and other constituents of plaques (58). Homocysteine also antagonizes the vasodilator properties of nitric oxide by the formation of S-nitrosohomocysteine, leading to endothelial dysfunction, the earliest stage in atherogenesis (12). Mechanisms leading to endothelial dysfunction by hyperhomocysteinemia depend probably on the generation of reactive oxygen species, decreased bioavailability of nitric oxide and concurrent elevation of asymmetric

dimethylarginine, a strong inhibitor of the nitric oxide synthase (59, 60).

Homocysteine may also have a harmful effect on mononuclear cells. Previous study demonstrated that homocysteine treatment caused a significant elevation of intracellular superoxide anion, leading to increased expression of chemokine receptor in monocytes (61). Homocysteine-stimulated superoxide anion production in monocytes is regulated through protein kinase C-dependent phosphorylation of p47<sup>phox</sup> and p67<sup>phox</sup> subunits of NADPH oxidase (62). Homocysteine alters the profile of cytokine/chemokine (interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha) production by endothelial cells and macrophages (63). Increased concentrations of homocysteine induce monocyte chemoattractant protein-1, interleukin-8 (16), and interleukin-6 accumulation in monocytic cells. After treatment with homocysteine, monocytic cells become more susceptible to endotoxin (64). In addition, It has been clearly demonstrated that homocysteine stimulates CCR2 (monocyte chemotactic protein-1 receptor) expression in monocytes, leading to an enhanced binding activity and chemotatic response (61). Increased superoxide anion production via NADPH oxidase may play an important role in homocysteine-induced inflammatory response during atherogenesis, and this altered cytokine/chemokine profile may be important in the inflammatory events that initiate or enhance the development of atherosclerotic lesions.

### 3.3. Hyperhomocysteinemia and vitamin supplement

Homocysteine can be lowered by supplement with folate and some B vitamins (65). Supplementation with these vitamins is inexpensive, safe, and effective in normalizing hyperhomocysteinemia (19, 66). In patients with hyperhomocysteinemia, vitamin treatment was associated with a decrease in cardiovascular disease risk in a controlled trial (67). In moderate hyperhomocysteinemia, vitamin supplement may lower homocysteine concentrations (68, 69). By contract, the NORVIT trial found no benefit of 0.8 mg/day of folate and 0.4 mg/day of vitamin B12 supplementation in survivors of myocardial infarction after a median follow-up of 40 months, and even suggested an increased risk of myocardial infarction, stroke or sudden death when 40 mg/day of vitamin B6 was added to the regime (22). The HOPE 2 trial in patients with vascular disease or diabetes who took folate, vitamin B6, and B12 supplements for 5 years also did not show a beneficial composite outcome in comparison with the control group (23). Thus, B vitamins supplements should not be used for prevention of atherothrombotic events in patients with mild to moderate hyperhomocysteinaemia (70).

Although the benefit of vitamin therapy seems ineffective and inconclusive, the evidence of total homocysteine to vascular damage remains abundant. Some may question if the homocysteine hypothesis is correct and homocysteine might be the bystander related to true atherogenic culprit. First, although folic acid is associated with thymidine metabolism leading to homocysteine lowering, folic acid itself could promote cell proliferation

(71) and the administration of folate, vitamin B6, and vitamin B12 had been reported to increase the risk of in-stent restenosis and the need for target-vessel revascularization after coronary stenting (34). Another possible mechanism is the complex methylation cycle of homocysteine. Homocysteine levels are reduced as a result of the enhancement of homocysteine methylation, which is promoted by the supplement of folic acid and vitamin B12. But this reduction can be associated with greater overall methylation potential, which can in turn increase cell proliferation, modify gene expression, and adversely affect endothelial function (72). Those are the possible explanations why the decreased serum level of homocysteine after vitamin supplement is not associated with the reduction of clinical events. Further exploration of homocysteine hypothesis and alternative approaches to reducing homocysteine concentration may be necessary.

## 4. ANTIOXIDATIVE AND ANTI-INFLAMMATORY STRATEGIES

### 4.1. Antioxidant supplement

The problem of homocysteine toxicity has attracted a great deal of interest. At molecular level several potential mechanisms were proposed, including mechanisms involving formation of reactive oxygen species (73). At cellular level pathological role of homocysteine seems to be associated with an alteration of endothelial cells function. Endothelial cells play an important role in regulating and maintaining the health of the vascular system and are very sensitive even to a mild increase in homocysteine concentration (73, 74). Folate and other antioxidant vitamins play an important role in determining homocysteine levels and potentially in regulating endothelial function (75, 76). Pretreatment with vitamin C and E prevented the postprandial impairment in endothelial function in 20 healthy volunteers after acute increases in homocysteine levels after receiving oral loads of methionine and found impairments in endothelial function (77).

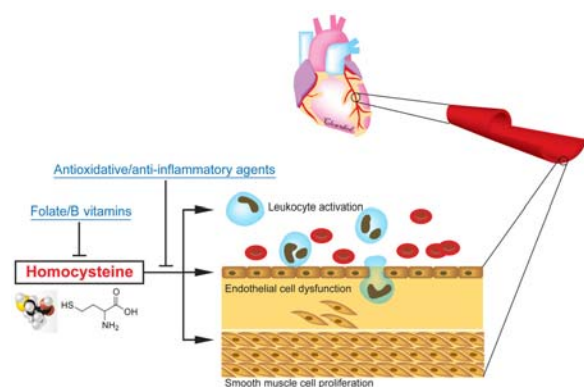
Folate supplementation is effective in lowering homocysteine levels, and several studies have examined the effects of folic acid on endothelial function in patients with hyperhomocysteinemia. In a randomized adults with angiographically diagnosed coronary artery disease with mild elevations in homocysteine levels ( $>9$   $\mu\text{M}$ ) (Three treatment groups: 5 mg folic acid daily, 5 mg folic acid + vitamin C and E, or placebo group). At baseline, all participants had impaired endothelial function as measured by flow-mediated dilation. Significant improvements were seen in flow-mediated dilation after 4 months of treatment with folic acid but not with placebo. However, the use of folic acid in combination with antioxidant vitamins did not significantly improve flow-mediated dilation (76). There remains great debate regarding the independent effects of antioxidant vitamins C and E on the endothelium, and studies have resulted in inconsistent findings. Indeed, vitamins C and E appear to have beneficial effects on the endothelium. Pretreatment with vitamins C and E before a high-fat meal decreased the postprandial impairment in endothelial function as measured by flow-mediated dilation

(78). High dosages of vitamins C and E in combination administered daily for 4 weeks was found to improve endothelial function as measured by forearm blood flow in chronic smokers (79). Nevertheless, folic acid may result in beneficial effects on the endothelium through mechanisms independent of lowering homocysteine, whereas studies on antioxidant vitamins C and E continue to yield conflicting results (75).

### 4.2. Anti-inflammatory strategy

Pro-inflammatory cytokines play critical role in inflammatory disease including atherosclerosis (80). Pathophysiological concentrations of homocysteine upregulate monocyte chemoattractant protein-1 and interleukin-8 expression and secretion in cultured endothelial cells suggesting that homocysteine may influence vascular disease by promoting leukocyte recruitment (15). Homocysteine also induces expression of monocyte chemoattractant protein-1 in smooth muscles cells (81) and monocytes (82). It has been shown that homocysteine induces expression of monocyte chemoattractant protein-1 and interleukin-8 through activation of nuclear factor-kappaB, a redox-sensitive transcription factor known to stimulate the production of chemokines, cytokines, hemopoietic growth factors, and leukocyte adhesion molecules (36), which are thought to be involved in atherogenesis and vascular inflammation (83, 84).

Hyperlipidemia is the term for a number of conditions of dysregulated lipid metabolism which require different treatment regimens. Large-scale randomized trials have shown that lipid-lowering with 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) reduce relative risk for cardiovascular events or death both in primary and in secondary prevention (85). Therefore, statins are widely used for the treatment of hypercholesterolemia. Statins are widely used for the prevention of cardiovascular disease through the reduction of total cholesterol and especially low-density lipoprotein-cholesterol. With respect to homocysteine, they have mainly been used as comparison to fibrates during short-term studies (86-89). In the prospective AFCAPS/TexCAPS trial, a small reduction of homocysteine during one year of treatment with lovastatin was observed (90), however, this finding was statistically significant, but the biological meaning of homocysteine reduction of  $-0.4$   $\mu\text{M}$  may be questioned. From these and other studies, it can be concluded that statins do not influence homocysteine concentrations significantly. Furthermore, the concurrent administration of statins and vitamins was investigated in a pilot study of the Study of the effectiveness of additional reductions in cholesterol and homocysteine (SEARCH) trial (91), revealing that there is obviously no effect of the statin component, as the reduction of plasma homocysteine levels was similar in the vitamin group and the vitamin plus statin group. More recently, the results of the SEARCH trial indicated that simvastatin 80 mg daily is not more efficacious than simvastatin 20 mg daily in the secondary prevention of adverse cardiovascular events in post-myocardial infarction patients. High-dose statin therapy is also associated with a



**Figure 1.** Schematic representation of possible mechanisms in homocysteinemia-induced atherosclerotic vascular dysfunction.

significantly higher incidence of myopathy, when compared with low-dose therapy. Further, homocysteine lowering with folic acid and vitamin B12 supplementation is also not associated with a reduction in cardiovascular events in this population (Presented at American Heart Association 2008). These results refute data from observational studies that suggested a possible beneficial effect on cardiovascular outcomes with homocysteine lowering. SEARCH should provide reliable evidence about the efficacy and safety of prolonged use of more intensive cholesterol-lowering therapy and, separately, of folate-based homocysteine-lowering therapy in a high-risk population (92). On the other hand, homocysteine is increased by administration of fibrin acid derivatives. Statins do not influence homocysteine levels, while the effect of nicotinic acid and n3-fatty acids is less clear. Concurrent vitamin administration with fibrates can attenuate the homocysteine increase substantially (93).

## 6. SUMMARY AND PROSPECTIVE

Homocysteine is a pro-thrombotic factor, vasodilation impairing agent, pro-inflammatory factor, and endoplasmic reticulum-stress inducer (74). However, the evidence shows that B vitamins supplements could not be used for the prevention of atherothrombotic events in patients with mild to moderate hyperhomocysteinemia. Our previous findings indicate that homocysteine could enhance the endothelial adhesiveness to activated mononuclear cells from coronary artery disease patients, which was obscure with less activated mononuclear cells from healthy subjects, suggesting the significant role of mononuclear cells activation in homocysteine-induced endothelial activation. On the other hand, treatment with statins similarly attenuated homocysteine-induced endothelial adhesiveness to activated mononuclear cells from coronary artery disease patients by reducing vascular cell adhesion molecule-1 expression, indicating the novel endothelial protection effects of statins in the presence of homocysteine (36, 37).

Our previous study also showed that the pre-existence of mononuclear cell activation was critical to and

required for homocysteine-induced endothelial adhesiveness (37). It may partially explain the clinical findings that reduction of plasma homocysteine can prevent future cardiovascular events in coronary artery disease patients after coronary intervention where monocytes are activated during the procedures (33). Given the current evidence of failure to prevent clinical cardiovascular events by solely reducing plasma homocysteine level, the alternative strategy directly targeting on endothelial or mononuclear cells protection may deserve to be mentioned for homocysteine-associated atherosclerotic diseases (94). Statins could attenuate homocysteine-induced endothelial adhesiveness by inhibiting the expression of endothelial vascular cell adhesion molecule-1, suggesting the effect of statins on direct endothelial protection. Interestingly, homocysteine was recently shown to induce 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase expression in vascular endothelial cells, providing the potential drug-specific mechanisms of statins for homocysteine-related atherosclerosis (95).

It has been suggested that homocysteine may induce vascular damage and can be a risk factor for atherosclerotic disease. In the presenting paper, we reviewed the available evidence concerning the association between plasma homocysteine and cardiovascular disease, with the objective of discussing the pertinence of screening, treatment, and prevention of hyperhomocysteinemia-related cardiovascular disease. Homocysteine, by inducing endothelial vascular cell adhesion molecule-1 expression, can enhance endothelial adhesiveness to mononuclear cells from coronary artery disease patients but not to that from healthy subjects, suggesting the requirement of mononuclear cells activation in homocysteine-related atherogenesis. The inhibition of smooth muscle cell proliferation, endothelial dysfunction, and mononuclear cell activation by anti-inflammatory and/or antioxidative drugs/agents may serve as a potential therapeutic strategy for hyperhomocysteinemia-related cardiovascular disease (Figure 1). Statins supplementation can directly reduce homocysteine-induced endothelial adhesiveness by inhibiting the expression of endothelial vascular cell adhesion molecule-1 expression, suggesting a novel vascular protective effect of statins in the presence of homocysteinaemia.

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**Send correspondence to:** Jaw-Wen Chen, Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 112, Taiwan, Tel: 886-2-28757125, Fax: 886-2-28740920, E-mail: jwchen@vghtpe.gov.tw

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